FBS11- Quantitation by Real-Time PCR Using Quantifiler Duo

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1. Scope

1.1. This procedure is used to quantitate the amount of amplifiable DNA in an extract.

2. Background

2.1. To establish the practices for documenting the examination of evidence to conform to the requirements of the Department of Forensic Sciences (DFS) Forensic Science Laboratory (FSL) *Quality Assurance Manual*, the accreditation standards under ISO/IEC 17025:2005, and any supplemental standards.

2.2. It is essential to assess the quantity of DNA present in an extract prior to amplification in order to obtain the most reliable results. The Quantifiler® Duo DNA Quantification Kit is a real-time polymerase chain reaction (rtPCR) assay designed to use a small portion of an extract to estimate the total quantity of amplifiable human DNA and male DNA present in the sample. The results obtained can aid in determining the quantity of extract needed for an STR reaction, the ratio of male to female DNA present in a sample and/or the presence of possible PCR inhibitors in the extract.

2.3. The kit consists of sequence specific primers, sequence specific TaqMan® probes labeled with fluorescent dyes, AmpliTaq Gold® DNA Polymerase and a reaction buffer containing dNTPs. The reagents are mixed with a small volume of sample extract and then placed in the AB 7500 for analysis. At the start of rtPCR thermal cycling, the TaqMan® probes are intact, enabling the quencher to suppress the fluorescence of the reporter dyes. During synthesis, the AmpliTaq

Gold® DNA Polymerase hydrolyzes the probes thereby separating the quenchers from the reporter dyes. A CCD camera enclosed within the AB 7500 records the resulting fluorescence. The amount of fluorescence emitted from each sample is proportional to the amount of DNA that is amplified through each cycle of the rtPCR process. The AB 7500 software plots the amount of fluorescence emitted with each cycle number from each dye, compares it to a series of standards, and estimates the amount of human and male DNA present in the extract. This estimate is based upon the value of the cycle threshold (C_T). The fewer cycles it takes to get to a detectable level of fluorescence, the greater the initial number of DNA molecules present in the sample extract.

3. Safety

3.1. Wear personal protective equipment (e.g., lab coat, gloves, mask, eye protection), when carrying out standard operating procedures.

3.2. Read Material Safety Data Sheets to determine the safety hazards for chemicals and reagents used in the standard operating procedures.

4. Materials Required

4.1. Quantifiler® Duo DNA Quantification Kit (FBQ31)

4.1.1. Note: It is important to minimize the number of freeze-thaw cycles for the kits. Keep the kits protected from direct exposure to light. Excessive exposure can affect the fluorescent probes. Each lot of kits must be evaluated prior to use. See QSOP 31 for information regarding the procedure for evaluation.

4.2. Quantifiler® Duo DNA Standards

4.2.1. Note: Standards should be stored from 2-5°C and expire after one week. See Quantifiler® Duo DNA Quantification Kit User's Manual for information regarding the procedure. Information regarding preparation of standards should be recorded in Quantifiler® Duo DNA Quantification Standards Log.

4.3. 96 well Optical Reaction plates
4.4. Optical adhesive covers
4.5. TE Buffer (FBR14)

5. Standards and Controls

5.1. Two duplicate sets of standards ranging from 0.023 ng/µl to 50 ng/µl are processed with each plate. These standards establish the standard curve which is utilized to estimate the quantity of DNA in each sample. The standard curve should be evaluated after each run using the following values:

	Acceptable Range	
	Human	Male
Slope	-3.77 to -3.06	-4.06 to -3.10
Y-Intercept	28.98 to 29.97	29.86 to 30.88
R squared	≥ 0.986	≥ 0.977
IPC Ct	≈28.51	≈29.37

5.2. NOTE: Quantitation values are subject to change depending on the Quantifiler® Duo kit lot quality control performance.

5.3. A Non-Template Control (NTC) consisting of master mix and 2µl TE Buffer is run once per plate. This sample should quantitate as a negative sample and the IPC should be in the appropriate range.

5.4. Present in the Master Mix and each sample, is an internal positive control (IPC). This control is added in a fixed concentration and should demonstrate that amplification occurred properly within each sample. If the C_T value is less than 20 or more than 30, it is possible inhibition may have occurred during the rtPCR process.

5.5. When setting up a rtPCR assay, DNA aliquots from questioned samples should be opened, aliquotted, and closed before opening and aliquotting the known samples. It is acceptable for both questioned and known samples to be run simultaneously on the real-time PCR assay.

6. Calibration

6.1. If the slope, y-intercept, and/or R^2 values are still out of range after two points have been deleted, all samples should be interpreted with caution or requantitated.

7. Procedures

7.1. Sample Preparation:

7.1.1. Refer to Chapter 3 in Quantifiler® Duo DNA Quantification Kit User's Manual

7.1.2. Record the sample set-up in the Quantifiler® Duo DNA Quantitation Set-Up Worksheet. Allow 16 spaces for the standards (2 duplicate sets) and 1 for the NTC.

7.1.3. Determine the amount of each reagent needed for the Master Mix by calculating the total number of samples, standards and blanks on the plate multiplied by the amount of each reagent needed per reaction. See chart below.

Component	***Volume per Reaction (µl)***
Primer Mix	*10.5*
Reaction Mix	*12.5*

10.5 x Number of Samples = Total Amount of Primer for Master Mix

12.5 x Number of Samples = Total Amount of Reaction Mix for Master Mix

7.1.3.1. Note: It may be necessary to add extra samples to the calculations to account for any loss during transfer steps.

7.1.4. If initial use of the kit, thaw the primer and reaction mixes completely. The primer mix may be vortexed and spun down to account for any settling of its constituents, however, the reaction mix must only be swirled gently.

7.1.5. Plate set-up should be completed in the hood. Add the calculated volumes to an appropriate container. Record the indicated lot numbers on the set-up worksheet.

7.1.6. Vortex and/or spin when possible.

7.1.7. Obtain a 96-well optical reaction plate and dispense 23µl of reaction mix into each sample well to be used.

7.1.7.1. Note: Extra care must be taken to be certain that the proper orientation of the plate is used without marking on the plate. Keep the reaction plate in a base at all times. DO NOT place the tray directly on the counter because it may interfere with subsequent fluorescence readings.

7.1.8. Add 2µl of each sample, standard, and control to the appropriate wells (the standards are run in duplicate).

7.1.9. Seal the plate with an optical adhesive cover. Avoid touching the center of the optical cover. Fingerprints or smudges can affect fluorescence leading to erroneous readings. If a print or smudge occurs, clean the area with ethanol and a kimwipe.

7.1.10. Centrifuge the plate at 3000 rpm for approximately 30 seconds to 1 minute to ensure all liquid is concentrated in the bottom of each well.

7.2. Turning on the Instrument:

7.2.1. Refer to Chapter 2 in Quantifiler® Duo DNA Quantification Kit User's Manual

7.2.2. Turn on the computer and log on.

7.2.3. Turn on the instrument by pressing the power button on the lower right front of the instrument.

7.2.4. Launch the AB Prism® 7500 SDS Software. The software will initialize and communicate with the 7500 instrument. When the connection is successful "Connected to 'Plate Name'" will be displayed in the status bar.

7.3. Creating a Plate Document:

7.3.1. Refer to Chapter 2 in Quantifiler® Duo DNA Quantification Kit User's Manual

7.3.2. In the software, select File > New to open the New Document Wizard Window. Make the following selections:

Assay – Absolute Quantitation

Container – 96-Well Clear

Template– Quantifiler Duo Template

(The operator, comments and default plate name fields are optional.)

The plate document may also be built without using the template.

7.3.3. Click Finish.

7.3.4. Type the sample names for each well location.

7.3.4.1. Note: It is possible to import a sample record from an appropriate format. Samples with identical names are treated as replicates by the SDS software. Results for replicate reactions are grouped together automatically for data analysis.

7.3.5. Select the Instrument tab.

7.3.5.1. Ensure the thermal profile is:

STAGE 1: 1 cycle, 50°C, 2:00 min.

STAGE 2: 1 cycle, 95°C, 10:00 min.

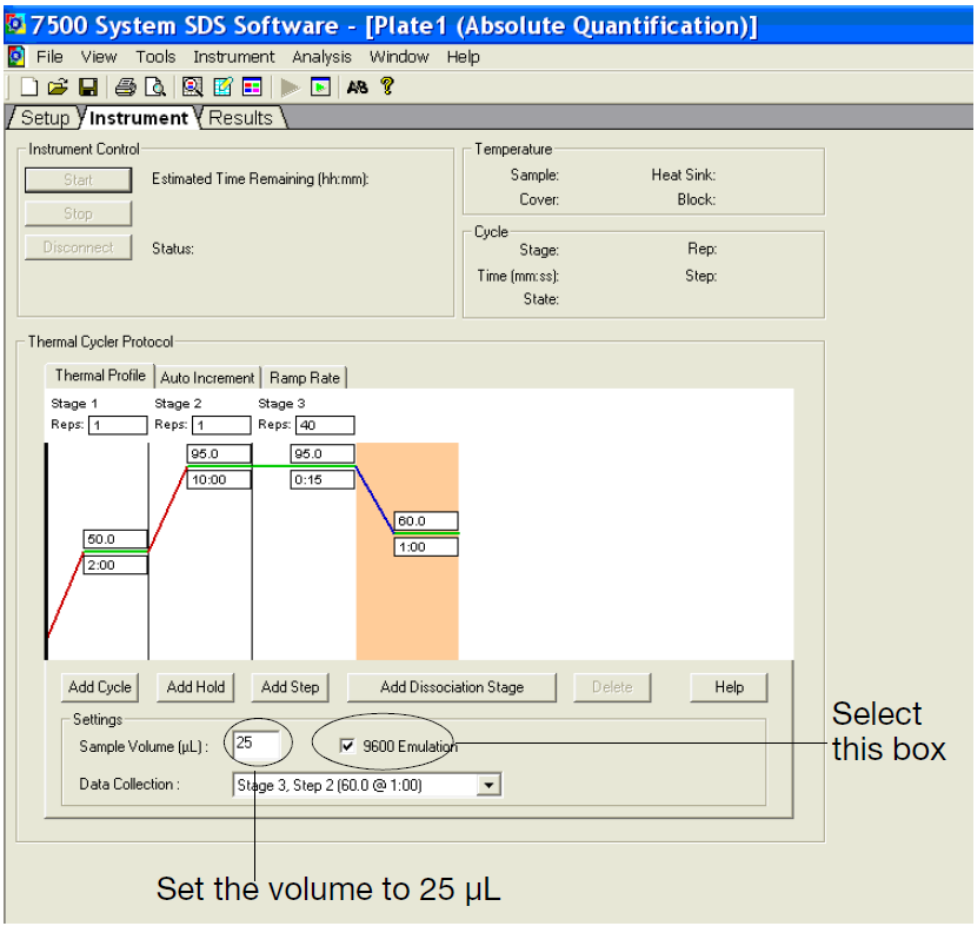
STAGE 3: 40 cycles, 95°C, Step 1, 00:15 min., Step 2, 60°C, 1:00 min

Ensure the Settings are:

25µl sample volume

Check 9600 Emulation

Data Collection – STAGE 3, Step 2 (60.0@1:00)



7.3.8. Enter a file name. Typically the file name includes the date and analyst initials. If an analyst runs more than one plate that day, a letter or number may be added.

7.3.9. For Save As type, select SDS Documents (*sds).

7.3.10. Click Save.

7.4. Running Samples:

7.4.1. Refer to Chapter 3 in Quantifiler® Duo DNA Quantification Kit User's Manual

7.4.2. Open the instrument by pressing on the depressed circle in the dark gray front of the instrument.

7.4.3. Once the plate holder tray opens, place the reaction plate into the precision plate holder.

7.4.4. Position the plate so that well A1 is in the upper left corner and the notched corner is in the upper right.

7.4.5. Gently push the plate holder closed.

7.4.6. In the 7500 SDS software, open the plate document that you set up for the run if it is not already open.

7.4.7. Select the instrument tab

7.4.8. Click Start

7.5. Analyzing a Run:

7.5.1. Refer to Chapters 4 and 5 in Quantifiler® Duo DNA Quantification Kit User's Manual

7.5.2. Open the plate document to analyze.

7.5.3. Verify the analysis settings by selecting Analyze > Analysis Settings.

Detector = All

Manual Ct Threshold = 0.200000

Manual Baseline Start (cycle) = 3, End (cycle) = 15

7.5.4. Select Analysis > Analyze or click the green arrow shortcut.

7.5.5. In the Results Tab, select the Standard Curve tab.

7.5.6. Evaluate the standard curve using the values listed in the Standards and Controls section of this SOP. The standard curve is a graph of C_T versus starting quantity of standards. The software calculates a regression line by calculating the best fit line for both the human and male detectors from the two sets of standards which were run. The following indicators are used to evaluate the quality of this line:

7.5.6.1. Slope - indicates the amplification efficiency of the standard reactions.

7.5.6.2. R^2 (correlation coefficient) - indicates the closeness of fit between the regression line and the individual data points.

7.5.6.3. Y-intercept - indicates the expected C_T value for a sample with a quantity of 1 ng/µl.

7.5.6.4. Note: 1 or 2 nonconcordant points may be removed from the standard curve (2 points CANNOT be removed from the same quantity standard). This omission should be noted on the setup worksheet.

7.5.7. Select the Report tab to evaluate the individual values for each sample. Sample quality can also be evaluated using the Amplification Plot tab. In the component view, the ROX™ passive reference should appear as a straight line across the x-axis. The NED™ labeled IPC should cross the threshold between cycles 20 and 30.

7.5.8. The internal positive control (IPC) is used to distinguish between true negative samples and PCR failures. The IPC is added to the reaction in a fixed concentration. The C_T for the IPC should range from 20-30. Use the following chart:

Human Quantitation Result	***IPC Detector Result***	***Interpretation***
No DNA present	*Amplification*	*No human DNA in sample*
No DNA present	*No Amplification*	*Invalid result; requantitate sample*
Very High Quantity DNA	*C_T value > 30*	*Disregard IPC result; or requantitate using a dilution*
Low Quantity DNA	*C_T value > 30*	*Partial PCR inhibition*

7.5.8.1. Note: Use caution when interpreting high quantity DNA samples. High levels of human DNA will compete with the internal positive control sequence PCR reactants and can lead to the control sequence not being efficiently amplified.

7.6. Record and print the appropriate results information. The data may be exported to an excel spreadsheet if necessary.

7.7. Discard plate and power down instrument.

8. Sampling

8.1. Not applicable

9. Calculations

9.1. All calculations are done within the software

10. Uncertainty of Measurement

10.1. When quantitative results are obtained, and the significance of the value may impact the report, the uncertainty of measurement must be determined. The method used to determine the estimation of uncertainty can be found in the *FSL Quality Assurance Manual – Estimation of Uncertainty of Measurement (Section 5.4.6).*

11. Limitations

11.1. When the sample IPC C_T is out of range, interpret with caution. Sample may be requantitated using a set of dilutions. Possible inhibition detected by the IPC is not an absolute indicator that there will be inhibition observed with amplification. As an optional step, a sample may be re-purified or concentrated a second time. (See FBS10 or FBS11.)

12. Documentation

12.1. Quantifiler® Duo DNA Quantification Standards Log

12.2. Quantifiler® Duo DNA Quantitation Set-Up Worksheet

13. References

13.1. Applied Biosystems. ABI Prism® 7500 Sequence Detection System User Guide. 2002.

13.2. Applied Biosystems. Quantifiler® Duo DNA Quantification Kit User's Manual. 2008.

13.3. Alfonina, I., Zivarts, M., Kutyavin, I., et. al. 1997. Efficient priming of PCR with short oligonucleotides conjugated to a minor groove binder. Nucleic Acids Research 25:2657-2660.

13.4. Green, R.L., Ines, C.R., Boland, C., and Hennessy, L.K. 2005. Developmental validation of the Quantifiler real-time PCR kits for the quantification of human nuclear DNA samples. Journal of Forensic Science. 50:809-825.

13.5. Higuchi, R., Dollinger, G., Walsh, P.S., and Griffith, R. 1992. Simultaneous amplification and detection of specific DNA sequences. BioTechnology 10:413-417.

13.6. Livak, K.J., Flood, S.J., Marmaro, J., Giusti, W., and Deetz, K. 1995. Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Appl. 4:357-362.

13.7. Quantifiler Duo DNA Quantification Kit and the 7500 Real-Time PCR Instrument Validation Report. Metropolitan Police Department, District of Columbia, 2009.

13.8. *Forensic Science Laboratory Quality Assurance Manual* (Current Version)

13.9. *FSL Departmental Operations Manuals* (Current Versions)

13.10. *FSL Laboratory Operations Manuals* (Current Versions)

13.11. *FBQ28 - Maintenance of the AB 7500 Real-Time PCR System (*Current Version*)*

13.12. *FBQ31 - Quality Control of Quantifiler® Duo DNA Quantification Kits (*Current Version*)*

13.13. *FBR14 - TE Buffer (*Current Version*)*

13.14. *FBS08 - Organic DNA Extraction (*Current Version*)*

13.15. *FBS09 - Differential Organic DNA Extraction (*Current Version*)*